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Inhibition of proliferation of human osteosarcoma cell line (MG63) by ajwain (*Trachyspermum ammi* L.) plant extract

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ABSTRACT

Trachyspermum ammi L. (TA), popularly called ajwain, is a well-known spice plant, with fruit pods that are used in Indian traditional medicine. Fruits of this plant were scientifically investigated for medicinal properties, but no studies are reported on the vegetative parts of the plant. Hence, the present investigation is aimed to evaluate the aerial parts of the plant for anti-proliferative and antioxidant effects. Methanolic extract (Me) of TA was subjected to sequential fractionation using the solvents in the increasing order of polarity viz. hexane (He), dichloromethane (Dcm), ethyl acetate (Ea), n-butanol (n-Bu), and water (Aq). Phytochemical analyses, DPPH radical scavenging assay, and *in vitro* antiproliferative activity on the MG63 osteosarcoma cell line (MTT assay) were performed. Preliminary phytochemical analyses revealed the presence of reducing and non-reducing sugars, amino acids, tannins, phenols, terpenoids, and saponins. Ethyl acetate fraction showed the highest amount of total phenolics (367.90 ± 0.02), followed by n-Bu (287.50 ± 0.04), Dcm (193.56 ± 0.07), Me extract (190.14 ± 0.07), Aq (142.20 ± 0.02) and He (80.74 ± 0.01) fractions. Ethyl acetate fraction exhibited higher DPPH radical scavenging activity ($IC_{50} = 116.74 \mu\text{g/mL}$) followed by n-Bu ($IC_{50} = 133.81 \mu\text{g/mL}$) fraction with a correlation to the higher amount of phenolics and flavonoids. However, maximum antiproliferative activity was exhibited by hexane fraction on MG 63 human osteosarcoma cell line which can be attributed to the presence of thymol, a monoterpene phenol present in ajwain. The current study indicates that the aerial parts of *T. ammi* Linn. can be considered as potential antioxidants and therapeutic against osteosarcoma.

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INTRODUCTION

Osteosarcoma is an aggressive bone cancer that primarily affects children and adolescents. High-grade osteosarcoma is currently treated by surgical resection, chemotherapy, and radiation therapy. Unfortunately, these treatments are reported to cause short-term and long-term side effects like cardio, renal, and myelotoxicities in many patients (Misaghi *et al.*, 2018). Hence, there is an urgent need to search for alternative remedies. Herbal medicines have been used for centuries to treat a wide range of ailments with few or no side effects because they are well tolerated, readily available, inexpensive, and considered non-toxic to normal human cells (Alam *et al.*, 2021).

Trachyspermum ammi L. Sprague (TA), commonly called ajwain, seeds of which were extensively used as therapeutic agents for

centuries for treating inflammatory diseases and digestive tract disorders by the practitioners of Ayurveda and Unani medicine. The plant is a member of the Apiaceae family, an aromatic annual herb with a striate stem that grows to a height of 2-3 feet. The small caraway-like fruit is the most used portion of ajwain and is very common in Indian savoury cuisine (Anilakumar *et al.*, 2009). Traditionally, the dried fruits are used to treat flatulence, atonic dyspepsia, diarrhoea, abdominal tumors, stomach-ache, piles, bronchial issues, lack of appetite, asthma, as well as amenorrhoea, and they are shown to possess antioxidant, antimicrobial, broncho-dilating, anti-helminthic, anti-filarial and cytotoxic activities. The chief bioactive constituents reported in the fruit pods are thymol, carvacrol, α -pinene, β -limonene, fiber, chromium, and calcium (Bairwa *et al.*, 2012). While studies have extensively been carried out on the seeds of ajwain, there are no investigations and reports on the aerial parts of ajwain plant.

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However, the leaves of TA are consumed by rural people in Anantapur district to treat gastritis and are also used by women after delivery to relieve joint pains. Hence, the present study was undertaken to identify phytochemicals in the extract and to assess bioactivities especially antioxidant and antiproliferative effects of aerial parts of ajwain plant.

MATERIALS AND METHODS

Collection and Authentication of Plant

Trachyspermum ammi Linn. seeds purchased in one lot were thoroughly cleaned and were sown in the campus garden under normal climatic conditions in August 2016 at Anantapur, Andhra Pradesh, India. Anantapur district is a semi-arid region in Rayalaseema, located at 14°41' N 77°36' E/14.68° N 77.6° E/14.68; 77.6 and is 335 m above the sea level. The inflorescence of the plant appeared four months after the date of sowing. Once the inflorescence set in, the plant is harvested, and only the aerial parts of the plants are taken for the study. The herbarium specimen of the plant was identified at Sri Krishnadevaraya University, Anantapur, by referring to the deposited specimen, and the herbarium voucher number was given as SSSIHL/BA&BA/2015-48720 (Figure 1). The aerial parts of the plant were washed thoroughly under running tap water, shade dried at room temperature for ten days. The dried parts were ground into a coarse powder using an electric blender, the powder was stored in an airtight container at 4 °C until further analysis.

Chemicals and Reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox), Pyrrolidine dithiocarbamate, Dimethyl sulfoxide, Rutin, Quercetin, Gallic acid, Thymol were purchased from Sigma-Aldrich (Bangalore, India). Fetal bovine serum, Dulbecco's modified Eagle's medium, 5-diphenyl-2-tetrazolium bromide, penicillin G, and streptomycin were obtained from Hi-Media (Mumbai, India). All other solvents and chemicals were from Sisco Research Laboratories (Mumbai, India) and Merck Millipore (Bangalore, India).

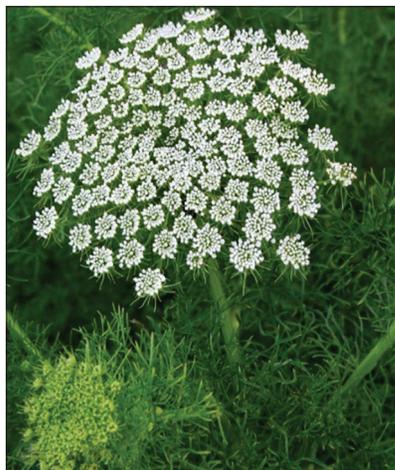


Figure 1: *Trachyspermum ammi* L. plant with inflorescence

Extraction of Plant Material

The dry plant powder (500 g) was subjected to extraction by percolation method with 80% methanol (Me) (1:1, w/v) thrice at room temperature. The whole extract was concentrated using a rotary vacuum evaporator (Heidolph, Germany) at 45 °C until the solvent was evaporated entirely. As shown in figure 2, a part of the methanolic extract (Me) was kept for analysis. The residue was mixed with distilled water and fractionated successively with different solvents in the increasing order of polarity viz. hexane (He), dichloromethane (Dcm), ethyl acetate (Ea), n-butanol (n-Bu), and distilled water (Aq). The fractions were evaporated to dryness, lyophilized, and stored in amber glass vials at 4 °C until further analysis. The extract recovered was weighed, and the percentage yield was calculated using the following equation:

$$\text{Extract recovery \%} = \left\{ \frac{\text{Amount of extract (g)}}{\text{Amount of plant sample (g)}} \right\} \times 100$$

Phytochemical Analysis

Qualitative Analysis

The presence of various phytochemicals in the methanolic extract and the fractions was determined by using standard tests. Carbohydrate derivatives were detected using Molisch, Benedict's, Barford's and Fehling's tests (Ramakrishnan *et al.*, 2004). Amino acid derivatives were identified using Ninhydrin, Millon's, Hopkin's cole, Ehrlich's, Pauly's and Nitroprusside tests (Ruthman, 1970). Flavonoids were detected using sodium hydroxide, sulphuric acid and sodium acetate tests. Tannins and phenols were identified using potassium ferricyanide, potassium dichromate tests and ferric chloride tests respectively (Harborne, 1973). Terpenoids by Salkowski test, steroids by Liebermann-Burchard's test and saponins by Foam's test were determined (Sofowora, 1993). Qualitative assessment was made based on the color development in various tests for phytochemicals and represented as +++ for large amounts, ++ for moderate, + for traces and – for not detected.

Quantitative Analysis

Estimation of total phenolic content (TPC)

Total phenolic content was determined by Folin-Ciocalteu method (Noreen *et al.*, 2017). To 500 µL of various samples, 2.5 mL of FC reagent (1:10) and 2.5 mL of 7.5% sodium carbonate solution were added, mixed, and incubated for two hours. The absorbance of the blue color developed was measured at 765 nm in a colorimeter against a blank. Gallic acid ranging from 50-500 µg/mL was used as standard. Total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g dried plant extract.

Detection of total flavonoid content (TFC)

The total flavonoid content of test samples was estimated by the aluminum chloride method (Pełal & Pyrzyńska, 2014). To 0.5 mL

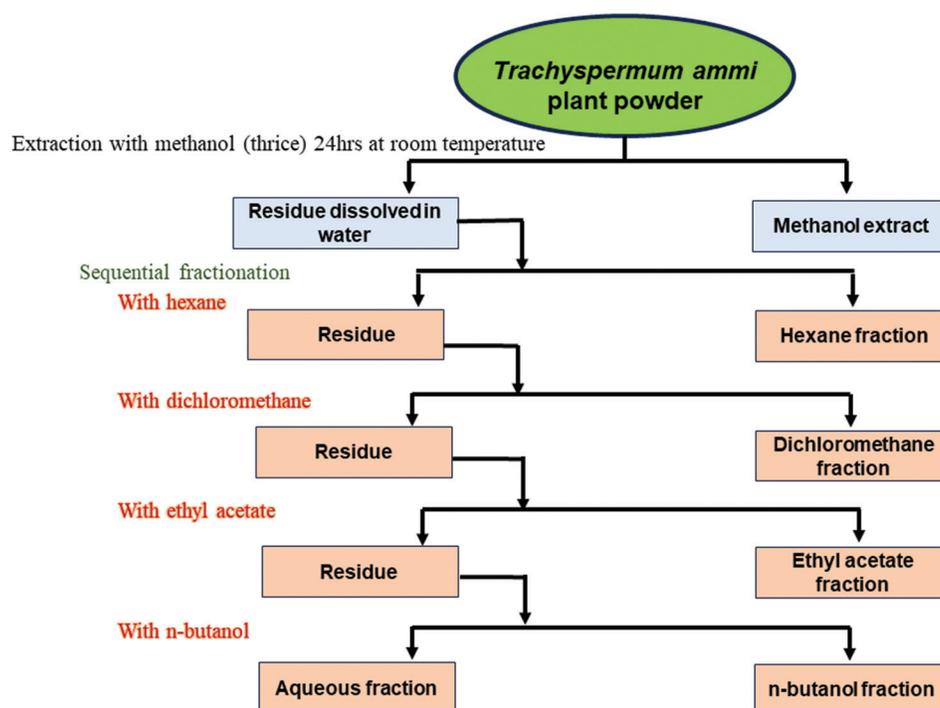


Figure 2: Preparation of methanolic extract and sequential fractionation

of various test samples, 1.5 mL of methanol, 0.1 mL of aluminum trichloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added and incubated at room temperature for 30 min. The absorbance of the color developed was read at 418 nm in a colorimeter. Rutin was used as standard at concentrations ranging from 100-500 $\mu\text{g/mL}$, and total flavonoid content was expressed as mg of Rutin equivalents (RE)/g dried plant extract.

Estimation of tannins

The total amount of tannins was quantified by using Folin-Ciocalteu (FC) method given by Govindappa *et al.* (2011). Tannic acid at a concentration ranging from 20-500 $\mu\text{g/mL}$ was used as standard. To 0.1 mL of test samples, 7.5 mL of distilled water, 0.5 mL of FC reagent, and 1 mL of 35% Na_2CO_3 were added, incubated for 30 min, and absorbance was measured at 700 nm in a colorimeter. All the determinations were done in triplicates. The tannin content of the samples was calculated from the calibration curve plotted using the absorbance values obtained for the standard. Results were reported as mg of Tannic acid Equivalents (TA)/g dry plant extract.

Assay of antioxidant Activity by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Method

The technique is a simple, quick, stable, and sensitive method for determining the antioxidant activity of a chemical or plant extract. Upon reacting with the H^+ donating antioxidant, DPPH gets reduced to hydrazine, bringing purple to yellow color, measured at 515 - 517 nm (Kedare & Singh, 2011). Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH *) radical by the

samples was evaluated in 96 well plates with slight modifications (Blois, 1958). To 50 μL of plant extract and its fractions (5-200 $\mu\text{g/mL}$), 150 μL of DPPH (0.1 mM in methanol) was added, the reaction mixture was shaken well and incubated at 37 $^\circ\text{C}$ for 30 min. in dark, the absorbance of the reaction mixture was measured at 517 nm using a Varioskan LUX Multi-mode Microplate Reader (Thermo Fisher Scientific, USA). Trolox was used as a positive control. Radical scavenging efficiency of the extracts was expressed as IC_{50} , the concentration of the sample required to scavenge 50% of DPPH * . The IC_{50} values were calculated using linear regression of plots.

The scavenging of DPPH by plant extracts was calculated using the following equation:

$$\text{DPPH}^* \text{ radical scavenging activity (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

Where A_c is the absorbance of the control (50 μL of methanol and 100 μL of DPPH solution), and A_t is the absorbance of the test sample. Results were obtained for triplicate samples.

Cell Proliferation Assay

Culturing of cell line

MG63 cell line [Osteosarcoma, from National Centre for Cell Science (NCCS)] was cultured using Dulbecco's Minimum Essential Medium (DMEM) L-Glutamine, Sodium bicarbonate, 10% Fetal Bovine Serum (FBS), 1% Antibiotic-Antimycotic (ABAM) (Penicillin, Streptomycin, Amphotericin) (Invitrogen, Thermo Fisher Scientific Inc). Cells were passaged

when the plate reached confluence, which is generally 24-36 hrs. Cells were cultured at 5% CO₂ and 37 °C temperature for growth and maintenance.

To understand the effect of methanolic extract (Me) of the plant and solvent fractions on Osteosarcoma (MG63) cells, MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) assay was carried out (Mosmann, 1983). Cell viability is generally measured by the capacity of the living cells to convert MTT to purple-colored formazan crystals. Once the culture plate reached confluence, the cells were harvested and seeded into a 96-well plate at 3x10³ per well. After 24 hrs of growth, Me extract and various fractions at different concentrations were added and further incubated at 5% CO₂ and 37 °C for 24 hrs.

The positive control used was Pyrrolidine dithiocarbamate (PDTc), a compound with known cytotoxicity, negative control was medium with untreated cells and vehicle control was untreated cells and medium with 0.1% DMSO. At the end of 72 hrs, MTT was added in the dark at 0.5 mg/mL concentration and incubated at 5% CO₂ and 37 °C for 3 hours. After this incubation step, the medium with MTT was removed and 100 µL DMSO was added to each well. DMSO dissolves the formazan crystals and gives light to a dark purple colour solution, based on the number of living cells. The percentage of cell viability was determined by measuring the absorbance of the wells at 570 nm in a multiplate spectrophotometer.

$$\% \text{ Cell viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100\%$$

Statistical Analysis

All the experiments were conducted in triplicates independently, and the results were expressed as mean ± SEM. The data were analysed using a one-way analysis of variance followed by F test (Gupta, 2014) in MS excel, and mean values were considered statistically significant at p<0.05.

RESULTS

Extract Recovery

Results indicated a notable difference in the yield with different solvents, as shown in Table 1, the highest yield was given by the n-Bu fraction (6.68%) followed by the Aq fraction and Ea with 4.64% and 3.96% respectively, Dcm fraction yield was 1.72% while the lowest yield was with, He fraction (0.4%).

Preliminary Phytochemical Screening

As depicted in Table 2, the present study showed the diversity of phytochemical constituents like carbohydrate derivatives, amino acids, flavonoids, phenols, steroids, triterpenoids, and saponins. Flavonoids were observed to be higher in Ea and n-Bu fractions. Steroids and terpenoids were predominant in He followed by Dcm, Ea, and n-Bu fractions.

Table 1: Extract recovery percentage of aerial parts of *Trachyspermum ammi* L. plant

Extract/Fractions	Weight (g)	Yield (%)
Methanolic extract (Me)	108.00	21.6
Hexane (He)	2.00	0.40
Dichloromethane (Dcm)	8.60	1.72
Ethyl acetate (Ea)	19.8	3.96
n-butanol (n-Bu)	33.4	6.68
Aqueous (Aq)	23.2	4.64

Quantification of total phenolics, flavonoids and tannins

Total phenolic content in Me extract and its fractions determined in this study varied from 80.74 - 367.90 mg/g and is expressed as gallic acid equivalents (GAE). Ethyl acetate fraction showed the highest amount of total phenolics (367.90±0.02), followed by n-Bu (287.50±0.04), Dcm (193.56±0.07), and Me extract (190.14±0.07) (Table 3). The ethyl acetate fraction of the plant exhibited the highest flavonoid content (330.00±0.09), followed by the Dcm fraction (304.50±0.4). The plant samples of TA had relatively low levels of tannins compared to phenolics and flavonoids. However, the highest tannin content was seen in the Ea fraction (75.94±0.01) and Me extract (72.02±0.1).

DPPH Radical Scavenging Activity

As is evident from Table 4, all the samples concentration dependently scavenged DPPH radical, however, the Ea fraction (IC₅₀=116.74) of methanolic extract exhibited the highest radical scavenging activity, followed by n-Bu fraction (IC₅₀=133.81) compared to the other fractions.

Anti-Proliferative Effect

As indicated in Figure 3, all the samples exhibited concentration-dependent inhibition of cell proliferation in the MG63 cell line. Hexane fraction at 200 µg/mL, exhibited the least proliferation (37.1%) of cells followed by Dcm fraction (68.9%). The multicomponent Me extract exhibited 72.5% cell proliferation and an Ea fraction of 78.3% at 200 µg/mL. Thymol-treated cell line exhibited lesser proliferation as compared to the treatments. The positive control, pyrrolidine dithiocarbamate (NFκB inhibitor) had shown the least proliferation.

DISCUSSION

Medicinal Plants have traditionally been used as a substantial source of bioactive compounds all over the world. Nearly 40% of the drugs, including aspirin, and artemisinin originated from plants and 80% of the human population still relies on traditional medicine for primary healthcare. Indigenous practitioners employed plant crude extracts or pure compounds to treat many diseases, including neurodegenerative disorders and cancer (Rana et al., 2021; Ayeni et al., 2022). The key aim of testing the cytotoxic activity of crude plant extracts is to isolate bioactive compounds that can be used as lead molecules in developing anti-cancer drugs or to develop crude extract into a formalized herbal medicine that can be used as a semi-chemo preventive agent (Iqbal et al., 2017).

Table 2: Qualitative screening of Me extract, He, Dcm, Ea, n-Bu and Aq fractions of Me extract of aerial parts of *Trachyspermum ammi* L. for phytochemicals

S. No.	Active compounds	Tests	Plant Extract and fractions					
			Me	He	Dcm	Ea	n-Bu	Aq
1	Carbohydrate derivatives	Molisch test	-	-	+++	++	+++	+++
		Benedict's test	+	+++	+++	++	-	++
		Barford's test	+++	+++	-	-	-	++
		Fehling's test	+++	++	+++	+	-	+++
2	Amino acid derivatives	Ninhydrin test	++	+	+	-	+++	+++
		Millon's test	++	++	++	++	+++	+
		Hopkin's cole test	-	-	-	-	-	-
		Ehrlich test	-	-	-	-	-	-
		Pauly's test	+	++	++	++	+++	-
		Nitroprusside test	-	-	-	-	-	-
		Sodium hydroxide test	+++	++	++	+++	+++	++
3	Flavonoids	Sulphuric acid test	++	++	+++	+++	+++	+++
		Sodium acetate test	++	++	++	+++	+++	+++
		Potassium ferricyanide test	+	-	+	+	+	-
4	Tannins	Potassium dichromate test	+	-	+	+	+	+
		Ferric chloride test	+	+	+++	++	+++	+
4	Phenols	Liberman- Buchard test	++	+++	++	++	++	+
		Salkowski test	++	++	+++	+++	++	+
5	Steroids and triterpenoids	Foam's Test	+	+	+	+	+	+

Me: Methanolic extract, He: Hexane fraction, Dcm: Dichloromethane fraction, Ea: Ethyl acetate fraction, n-Bu: N- butanol fraction, Aq: Aqueous fraction of methanolic extract. +++ Present in large amounts, ++ Moderate, + Traces, - Not detected

Table 3: Phytochemicals in Me extract, He, Dcm, Ea, n-Bu, and Aq fractions of Me extract of aerial parts of *Trachyspermum ammi* L.

Sample (500 µg/mL)	Phytochemicals		
	Total Phenolics mg/g GAE	Total Flavonoids mg/g RE	Total Tannins mg/g TAE
Me	190.14±0.07	137.84±0.1	72.02±0.1
He	80.74±0.01	133.92±0.1	30.19±0.1
Dcm	193.56±0.07	304.50±0.4	41.96±0.2
Ea	367.90±0.02	330.00±0.09	75.94±0.01
n-Bu	287.50±0.04	159.40±0.1	48.48±0.1
Aq	142.20±0.02	77.04±0.1	43.26±0.1

Me: Methanolic extract, He: Hexane fraction, Dcm: Dichloromethane fraction, Ea: Ethyl acetate fraction, n-Bu: N- butanol fraction, Aq: Aqueous fraction of methanolic extract. Values are mean±SEM of three replicates

In the present study, the extraction of aerial parts of TA by percolation method, recovered most of the bioactive components (Table 2) because it was carried out at room temperature without using heat to protect thermolabile compounds (Singh & Ahmad, 2017). The percentage yield and isolation widely depend on the method of extraction, nature of phytochemicals, particle size etc. Results indicated a significant difference in the yield with different solvents because differences in the polarity of solvents could result in a wide range of bioactive compound levels in the extract (Truong *et al.*, 2019). Conventional preliminary phytochemical screening is an important stage in detecting major bioactive principles in plants (Wrona *et al.*, 2019). Results of the qualitative analysis revealed the presence of flavonoids, phenolics, alkaloids, terpenoids, and tannins in aerial parts of TA similar to that of phytochemicals observed in the fruit pods of TA (Bashyal & Guha, 2018). Among these, polyphenols are considered naturally occurring antioxidants that are attributed to possess oxygen quenching, reducing, and metal

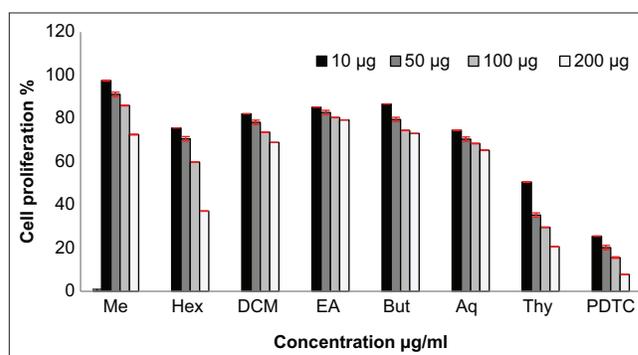


Figure 3: Cell proliferation (%) shown by Me extract, solvent fractions, thymol, and PDTTC-treated MG63 cell lines (Me: Methanolic extract, He: Hexane fraction, Dcm: Dichloromethane fraction, Ea: Ethyl acetate fraction, n-Bu: n- butanol fraction, Aq: Aqueous fraction, Thy: Thymol, PDTC: Pyrrolidine dithiocarbamate). Values are mean ±SEM of three replicates: (p < 0.05)

chelating abilities and are reported to positively affect human health (Jomová *et al.*, 2019). In this study, total phenolics and flavonoids were found to be higher in Ea fraction due to which Ea fraction effectively reduced purple-colored 2,2-diphenyl-1-picryl-hydrazyl (DPPH) organic free radical to yellow-colored 2,2-diphenyl-1-picryl-hydrazine and hence proved its antioxidant activity. Similarly, high radical scavenging activity exhibited by ethyl acetate extract of *T. ammi* L. seeds compared to the other extracts (Goswami & Chatterjee, 2014). Hence these studies revealed that both seeds and aerial parts of the Ea fraction possess active constituents that donate hydrogen to a free radical reaction, leading to the discoloration of the purple color of DPPH. The scavenging activity of Ea fraction is dependent on the extractable phenolics and flavonoids, as observed by qualitative and quantitative phytochemical screening in the present study. These findings also suggest higher solubility of

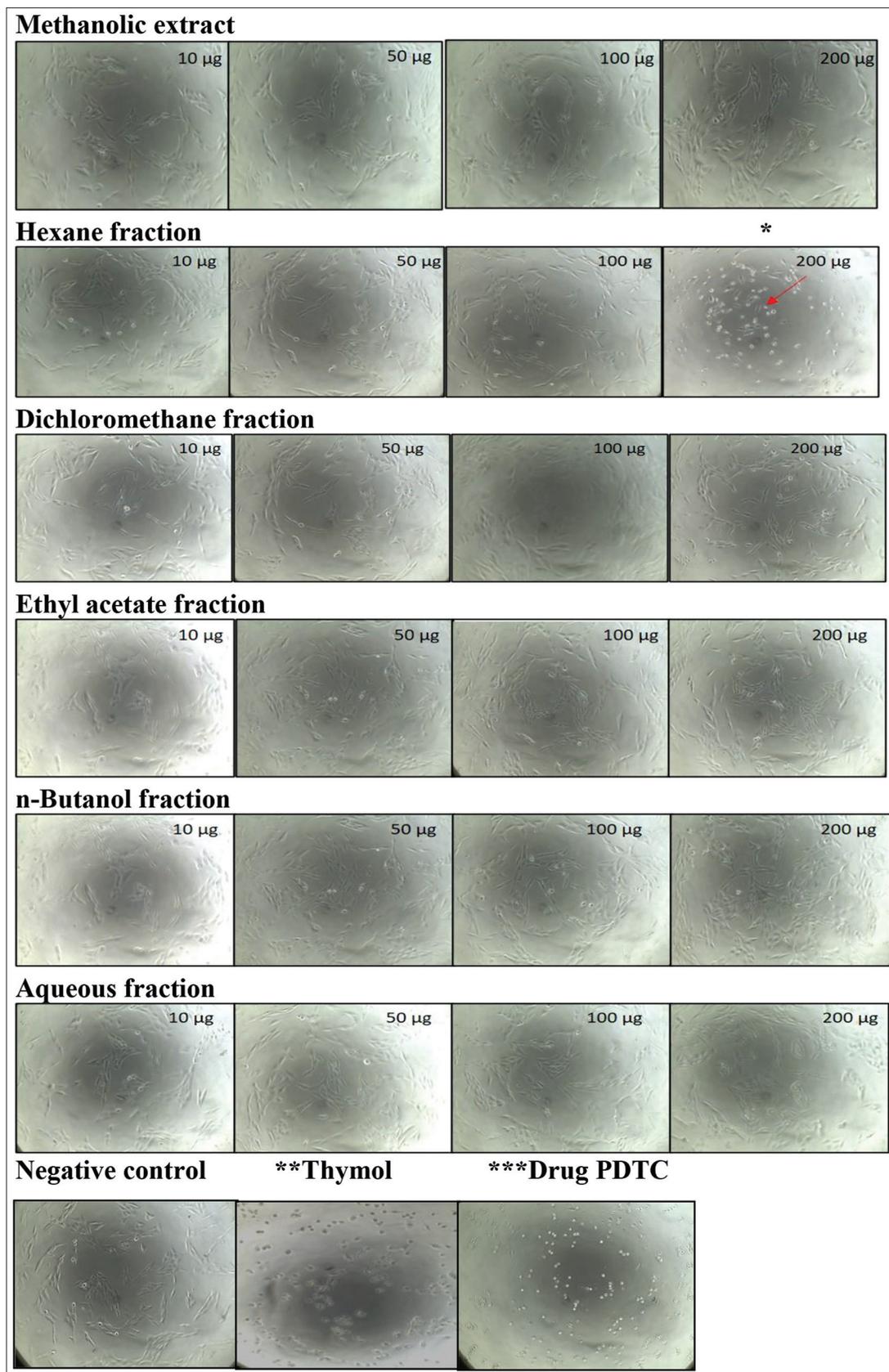


Figure 4: Anti-proliferative effect exhibited by Me extract, He, Dcm, Ea, n-Bu and Aq fractions of Me extract of aerial parts of *Trachyspermum ammi* L. negative control, thymol, and drug on MG63 Osteosarcoma cell lines (*Hexane fraction (200 µg/mL) treated cell line, **Thymol-treated cell line, ***Pyrrolidine thiocarbamate-treated cell line)

Table 4: DPPH radical scavenging activity (%) of Me extract, He, Dcm, Ea, n-Bu and Aq fractions of Me extract of aerial parts of *Trachyspermum ammi* L. and positive control (Trolox)

Sample	Concentration ($\mu\text{g/mL}$)					
	5	10	50	100	200	IC ₅₀
Me	15.86 \pm 0.4	19.35 \pm 0.4	21.65 \pm 0.7	26.61 \pm 0.3	38.13 \pm 0.5	315.66
He	6.25 \pm 0.3	9.35 \pm 0.3	12.70 \pm 0.6	16.80 \pm 0.4	22.85 \pm 0.1	534.45
Dcm	7.83 \pm 0.6	13.67 \pm 0.8	17.55 \pm 0.3	25.17 \pm 0.1	35.02 \pm 0.5	381.70
Ea	19.36 \pm 0.1	24.78 \pm 0.2	34.22 \pm 0.2	46.29 \pm 0.2	70.40 \pm 0.6	116.74
n-Bu	10.91 \pm 0.5	22.25 \pm 0.8	30.48 \pm 0.2	43.52 \pm 0.4	65.32 \pm 0.3	133.81
Aq	7.30 \pm 0.6	10.60 \pm 0.5	15.48 \pm 0.5	22.44 \pm 0.1	30.51 \pm 0.8	309.12
Tro	26.13 \pm 0.3	35.70 \pm 0.5	52.55 \pm 0.5	80.23 \pm 0.2	89.09 \pm 0.5	51.76

Me: Methanolic extract, He: Hexane fraction, Dcm: Dichloromethane fraction, Ea: Ethyl acetate fraction, n-Bu: n- butanol fraction, Aq: Aqueous fraction, Tro: Trolox. Values are mean \pm SEM of three replicates

phenolics and flavonoids in the polar solvents like ethyl acetate resulting in higher antioxidant potential.

In many types of cancers, including osteosarcoma, the nuclear factor kappa B (NF- κ B) gene is hyper-activated, and the growing number of clinical studies proved that the phytomedicinal approach can serve as the best therapeutic alternative in its downregulation (Gong *et al.*, 2017; Saleh *et al.*, 2021). Therefore, NF- κ B signaling inhibitor ‘pyrrolidine thiocarbamate,’ was taken as a positive control in the cell proliferation assay.

In the present investigation, hexane fraction at 200 $\mu\text{g/mL}$ and thymol exhibited a prominent anti-proliferative effect compared to the other fractions (Figure 4). In particular, the previous findings emphasize that the main bioactive compound present in the hexane fraction (non-polar solvent) of ajwain seeds is Thymol (Anilakumar *et al.*, 2009; Rahimmalek *et al.*, 2017) and studies reported thymol to be an excellent anti-tumor agent against many cancers (Ramya *et al.*, 2017). Hence, the anti-proliferative effect shown by the hexane fraction in the present investigation is attributed to the presence of thymol, a monoterpene extracted by hexane from the aerial parts of ajwain plant.

The observations indicate that the non-polar and semi-non polar components of TA may have molecules involved in the activation mechanisms of one or more antiproliferative pathways compared to polar components extracted in polar solvents (Seresht *et al.*, 2019). Considering the above observations, it can be stated that aerial parts of TA plant possess potent antioxidants and anti-proliferative agents against osteosarcoma cell lines. However, the identification of bioactive compounds from potential fractions *viz.* n-hexane, and ethyl acetate fraction of methanolic extract is worthwhile to clarify the biochemical nature of compounds. Further investigations for applications of new natural antioxidants and elucidation of the chemical composition of phenolics, flavonoids, and terpenoids are warranted to establish the functionalities to find out the regulatory effect of bioactive compounds on the NF- κ B signalling pathway in the osteosarcoma cell line. This research represents the first report on the antiproliferative activity (*in vitro*) of *T. ammi* L. aerial parts.

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